Appendix C

(C1 – C12)

Appendix C1

CRE-Luc Reporter Assay

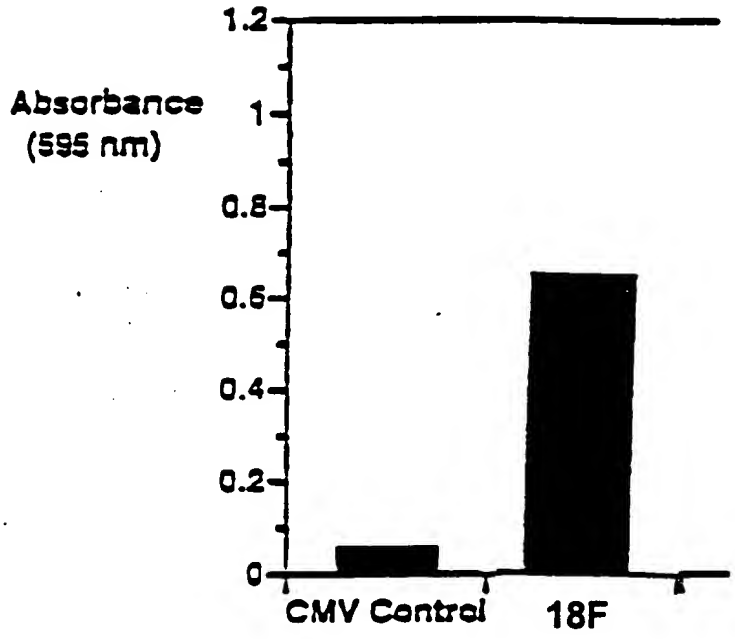
To generate a ß-galactosidase reporter containing multiple Gal4 binding sites, a Bgl II/ HindIII fragment was removed from the somatostatin promoter-containing plasmid 1.4(5xGal)CAT (Leonard, J. et al (1992) *PNAS USA* 89:6247-6251) and cloned into p ß gal-Basic (Promega). The Bgl II/ HindIII fragment contains a variant of the minimal somatostatin promoter (from -71 bp to +50 bp relative to the transcription start site) in which the core 4bp of the cAMP Response Element (-46 to -43) were replaced with 5 copies of the recognition sequence for the yeast transcription factor Gal4. When this reporter is co-transfected with an expression plasmid encoding a Gal4-CREB fusion protein, it is highly responsive to agents that increase the cAMP signaling pathway.

VIP2.0Zc is a cell line that has been stably transfected with the reporter gene ß-galactosidase under the control of a cAMP responsive VIP promoter (Konig et al. Mol. Cell.Neuro. 1991, 2, 331-337). The cell line was used here to indirectly measure the accumulation of intracellular cAMP. Approximately 2 million cells were plated in 6 cm plate the day before transfection. DNA (5 µg), for each receptor, was mixed with 2.5 ml serum-free DMEM containing 200 µg/ml DEAE dextran and 100 µM chloroquine, and added to a rinsed cell monolayer. After incubation for 90 min in a CO_2 incubator, the transfection medium was removed. The cells were washed with serum-free medium and supplemented with fresh complete medium. Twenty four hours after transfection, the cells were replated into 96-well plate at a density of 50 - 100 K per well and the ß-galactosidase activity was assayed 48 to 72 hours after transfection.

The assay buffer contained 100 mM sodium phosphate, 2 mM $MgSO_4$, 0.1 mM $MnCl_2$, pH 8.0. The cells were washed with PBS, and 25 µl /well of hypotonic lysis buffer consisting of 0.1 X assay buffer was added. Ten minutes later, 100 µl of assay buffer containing 0.5% Triton X-100 and 40 mM ß-mercaptoethanol was added to each well and incubation at room temperature continued for 10 minutes. The substrate solution containing 5 mg/ml chlorophenol red- ß-D-galactopyranoside (CPRG) in assay buffer was added at 25 µl/well and the plate was incubated at 37°C for 30 minutes before absorbance at 595 nm was measured with a plate reader.

18F was assayed using the foregoing system, and it was determined that 18F is constitutively active.

Appendix C2



Appendix C3

In situ Hybridization Protocol

1. ***Preparation of In Situ Probes***

The in situ probe DNA fragment of rat 18F was obtained by PCR based on the published rat 18F cDNA sequences. The sequences of the oligonucleotides utilized were as follows:

5'-GGAGAAGCTTCTGGCGGCGATGAACGCTAG-3' (5' oligo)

5'-ACAGGATCCAGGTGGCTGCTAGCAAGAG-3' (3' oligo).

The PCR condition utilized was as follows: the reaction condition utilized was 1X rTth DNA polymerase buffer II, 1.5 mM $Mg(OAc)_2$, 0.2 mM each of the 4 nucleotides, 0.228 μg rat genomic DNA, 0.25 μM of each primer (*see* below) and 1 unit of rTth DNA polymerase (Perkin Elmer) in 50 μl reaction volume. The cycle condition was 30 cycles of 94°C for 1 min, 55 °C for 1 min and 72 °C for 45 sec with a Perkin Elmer Cetus 2400 thermal cycler.

A 608 bp PCR fragment containing nucleotide -10 through to the middle of transmembrane domain 4 was digested with Bam HI and Hind III and was subcloned into Bam HI-Hind III site of pBluescript.

In situ probe sequences generated were as follows:

AAGCTTCTGGCGGCGATGAACGCTAGCGCCGCCGCGCTCAACGAGTCCCAGG
TGGTGGCAGTAGCGGCCGAGGGAGCGGCAGCTGCGGCTACAGCAGCAGGGACACCG
GACACCAGCGAATGGGGACCTCCGGCAGCATCCGCGGCGCTGGGAGGCGGCGGAGG
ACCTAACGGGTCACTGGAGCTGTCTTCGCAGCTGCCCGCAGGACCCTCAGGACTTCT
GCTTTCGGCAGTGAATCCCTGGGATGTGCTGCTGTGCGTGTCGGGGACTGTGATCGC
AGGCGAAAATGCGCTGGTGGTGGCGCTCATCGCATCCACTCCCGCGCTGCGCACGCC
CATGTTTGTGCTCGTGGGTAGTCTGGCCACTGCTGACCTGCTGGCGGGCTGTGGCCTC
ATCCTACACTTCGTGTTCCAGTACGTGGTGCCCTCGGAGACTGTGAGCCTGCTCATGG
TGGGCTTCCTGGTGGCGTCCTTCGCCGCCTCAGTCAGCAGCCTGCTCGCTATCACAGT
GGACCGTTACCTGTCCCTTTACAACGCGCTCACCTACTACTCGCGCCGGACCCTGTTG
GGCGTGCACCTCTTGCTAGCAGCCACCTGGATCC

18F probe was produced from a 450bp HindIII-ScaI fragment of the 18F receptor cloned into the HindIII-SmaI site of pBluescriptSK+. Riboprobes were produced using a T7 transcription system in a standard labeling reaction consisting of: 1μg of linearized plasmid , 2μl of 5x transcription buffer, 125μCi of ^{35}S-UTP, 150μM of GTP, CTP and ATP, 12.5mM dithiothreitol, 20U of Rnase inhibitor and 6U of appropriate polymerase. The reaction was incubated at 37°C for 90 min., labeled probe being separated from free nucleotides over Sephadex G-50 spin columns.

Appendix C3 cont'd

2. ***Tissue preparation***

Dissected tissue was frozen in isopentane cooled to -42°C and subsequently stored at -80 °C prior to sectioning on a cryostat maintained at -20°C. Slide-mounted tissue sections were then stored at -80 °C.

3. ***In Situ Hybridization Protocol***

Tissue sections were removed from the -80°C freezer and incubated with a 1 µg/ml solution of proteinase-K to permeabilize the tissue and inactivate endogenous RNase. After this treatment, sections were incubated in succession in water (1 min), 0.1 M triethanolamine (pH 8.0; 1 min), and 0.25% acetic anhydride in 0.1 M triethanolamine (10 min). The tissue was then washed in 2 x SSC (0.3 mM NaCl, 0.03 nM Na citrate, pH 7.2; 5 min) and dehydrated through graded concentrations of ethanol. Sections were then hybridized with 1.5 x 10^6 dpm of [^{35}S]UTP-labeled cRNA probes in 20 µl of a hybridization buffer containing 75% formamide, 10% dextran sulfate, 3 x SSC, 50 mM sodium phosphate buffer (pH 7.4), 1 x Denhart's solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml sheared salmon sperm DNA. Tissue sections were covered with coverslips that were sealed with rubber cement. The slides were incubated overnight at 50°C. On the following day, the rubber cement was removed, the coverslips were soaked-off with 2 x SSC, and the tissue sections were washed for 10 min in fresh 2 x SSC solution. Single stranded probe not hybridized with endogenous mRNAs was removed by incubating the sections for 30 min in 200 µg/ml solution of RNase-A at 37°C. The tissue was then washed in increasingly stringent SSC solutions (2, 1 and 0.5 x SSC; 10 min each), followed by a 1 hr wash in 0.5 x SSC at 60°C. After this final wash, the tissue sections were dehydrated using graded concentrations of ethanol, air dried and prepared for detection by x-ray autoradiography on Kodak XAR-5 film.

4. ***Analysis***

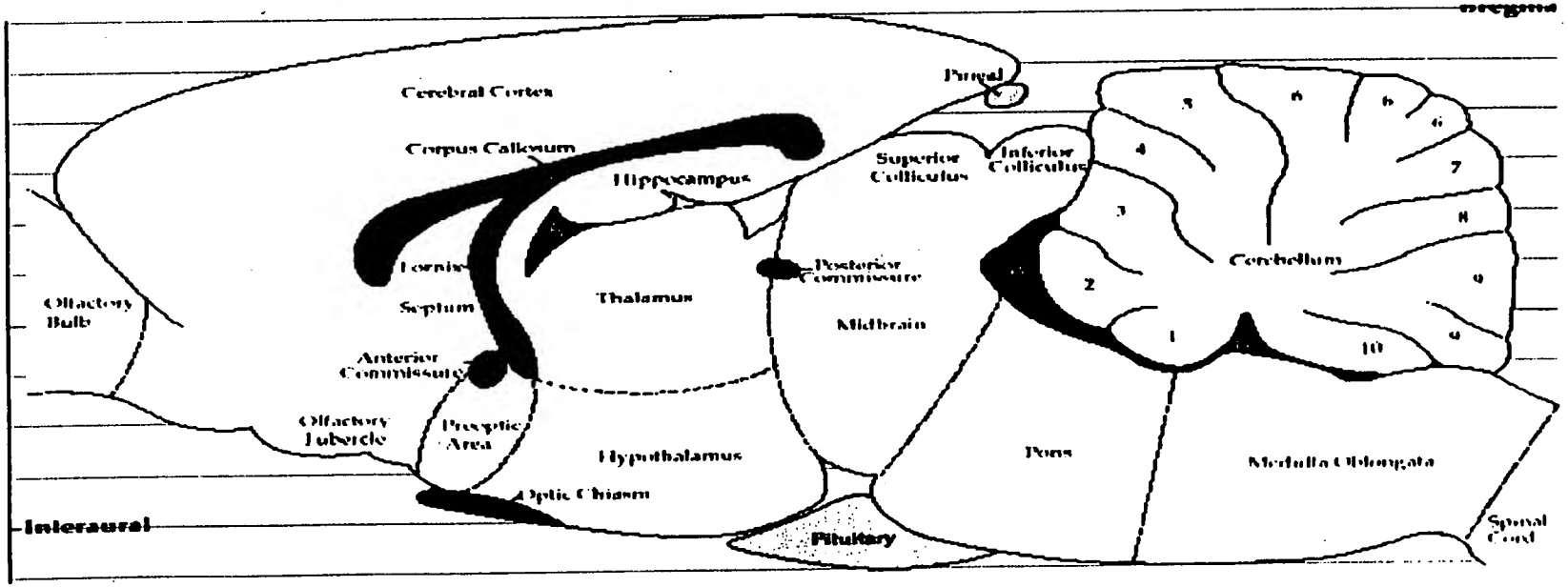
Utilizing the above protocol on normal male rats (Sprague-Dawley, Charles River), it was determined that 18F is expressed in the following areas of the brain: hypothalamus, hippocampus, nucleus accumbens, caudate and cerebral cortex. *See* **Appendix C4** for a representative tissue section (18F receptor is presented in the dark areas; **Appendix C5** provides a reference map of the rat brain.)

Appendix C6 provides a representative tissue section of 18F receptor expression in the lean Zucker animals; **Appendix C7** provides a representative tissue section of 18F receptor expression in the obese Zucker animals; and **Appendix C8** is a reference map of this section of the rat brain.

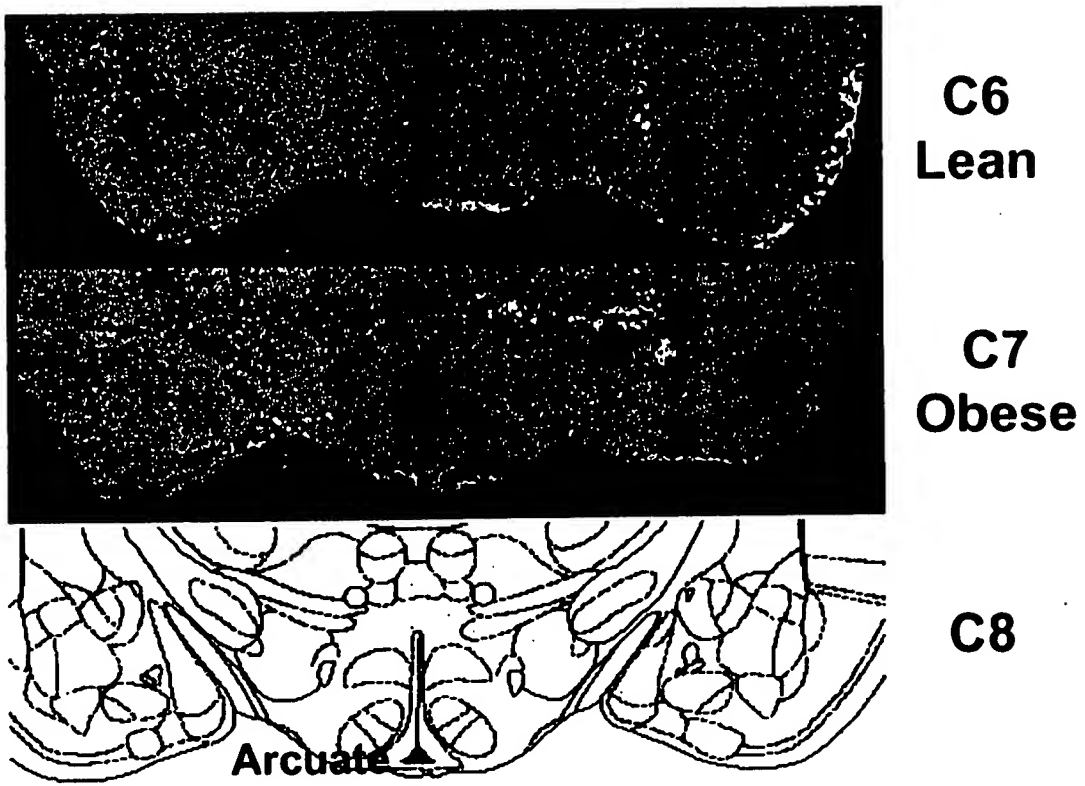
Appendix C

C4

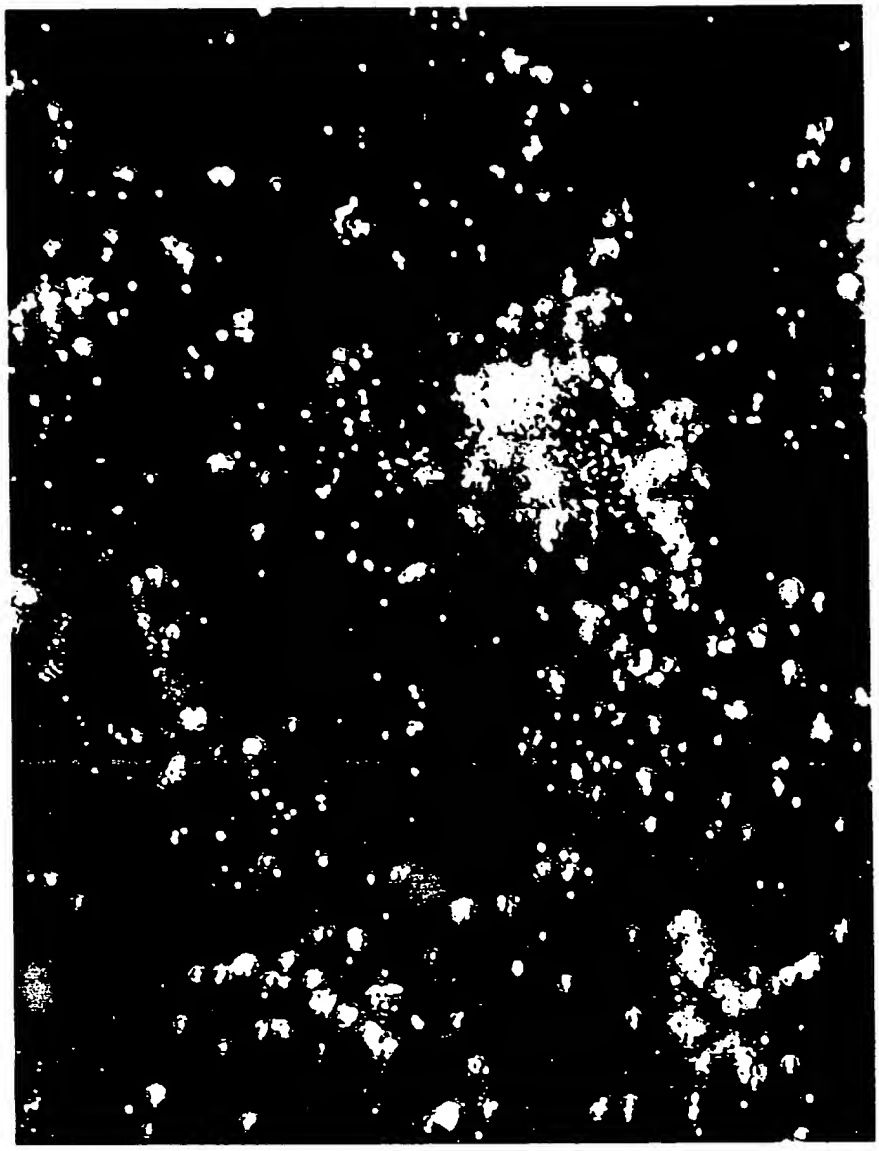
C5



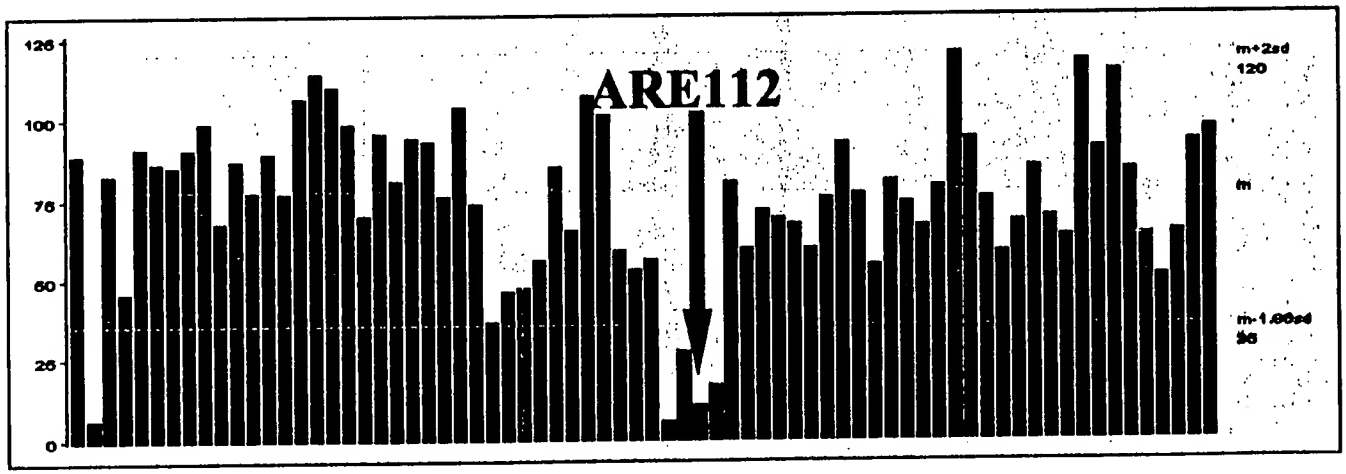
Appendix C



Appendix C9



Appendix C10



Appendix C11

In Vivo Assay Protocol

1. Food/Water Intake and Body Weight in 24-Hour Food-Deprived Animals Intracerebroventricular (ICV) Administration of ARE112

a. Surgery

Animals were prepared with an ICV cannula aimed above the lateral ventricle. For this surgery, animals were placed under general anesthesia using continuous inhalation of isoflurane and secured in a Kopf stereotaxic instrument. Surgery was performed in a dedicated surgery room, using sterile instruments, surgical gloves and aseptic procedures to prevent clinical infections. The surgical site was shaved and disinfected with betadine solution and alcohol. Animals were observed continuously for the level of anesthesia by testing for the animals' responses to tail- or paw-pinch. A cannula made of 23 gauge stainless steel tubing (7mm long) was lowered to a point 1mm above the ventricle, using coordinates: A/P –0.6 mm from bregma, M/L+/- 2.0 mm from bregma, D/V 3.2 mm below skull surface. The guide was anchored to the skull with three stainless steel screws and dental cement. Coordinates were based on stereotaxic atlas such as Paxinos and Watson sterotaxic atlas (Paxinos, G. and Watson, C, The Rat Brain, New York, Academic Press, 1982). After cannula implantation, a 30-gauge stainless steel dummy stylet was inserted into the cannula. At the conclusion of surgery, a heat source (heat lamp directed towards one half of recovery cage) was used to maintain body temperature while the animals recovered from anesthesia. Animals were allowed at least one week of recovery after surgery before ICV injection of compound was performed.

b. Administration of ARE112

Animals were food-deprived for 24 hours prior to administration of the compound.

For ICV injection, the dummy stylet was removed from the implanted cannula, and a 30-gauge stainless steel injector cannula containing ARE112 suspension in a 45% cyclodextrin solution was inserted to a depth 1.5mm beyond the ventral tip of the implanted cannula. The other (non-inserted) end of the injector cannula was attached to 60 cm of PE10 tubing containing the compound suspension, which was attached at the free end of tubing to a 25µl Hamilton syringe. Ten microliters of ARE112 suspension (containing, 0 and 100nmol) were then delivered via gentle and even mechanical pressure to the plunger of the Hamilton syringe. The volume of injection was verified by marks on the PE 10 tubing previously calibrated with a 10 µl Hamilton syringe. At the conclusion of injection, any fluid observed from the dorsal tip of the implanted cannula upon withdrawal of the injector cannula was noted, and the dummy stylet was inserted into the implanted cannula.

Data evidence that after six hours post-administration of ARE112 at all doses, animals consumed substantially less food (about 3 fold less food was consumed). See **Appendix C12** panel 1.

2. Food/Water Intake and Body Weight in 24-Hour Food-Deprived Animals Oral (PO) Administration of ARE112

Based upon the *in vivo* data developed, oral bioavailability of compound ARE112 was determined. The compound was administered by oral gavage at doses ranging from 0, 6.75, 13.5, 27 and 54 mg/kg. The data suggest that oral administration of ARE112 also decreases food intake in both food-deprived and non-food deprived rats. The effect of ARE112 was dose-dependent. In this assay, the vehicle-treated rats at 6 hours consumed about 14 grams of food while injected rats; particularly rats administered with 27 and 54mg/kg of ARE112, ate about 10 and 8 grams, respectively. See, **Appendix C12**, panel 2.

3. Basal Food/Water Intake and Body Weight in Non-Food Deprived Animals

Animals were also observed during their most active period (dark cycle), and both basal food and water intake were measured. In this test, animals were not deprived of food but were instead observed for normal activity.

Animals were orally administered 18F112 at 0, 6.75, 13.5, 27 and 54 mg/kg, 30 min prior to the beginning of their dark cycle (*i.e.* 6:30 pm) and were then exposed to standard rat chow pellets (*i.e.* 30 min after compound administration) and observed for a period of 15.5 hr post administration. In a second assay, rats were treated with 18F112 by oral gavage but were not food deprived. Data evidence an even greater decrease in food intake, such that at 18 hours post administration of the compound, the vehicle rats had consumed about 18 grams of food while the injected rats (at all doses) ate 5grams or less; about a four fold decrease in food consumption when treated with compound ARE112. (*See*, **Appendix C12**, panel 3). These data support the conclusion that ARE112 is orally active.

4. Effect of ARE112 on Motor Function

The effect of ARE112 on motor function was also examined. Motor function was assessed by using automated locomotor activity cages. The rats were placed in a standard rodent cage

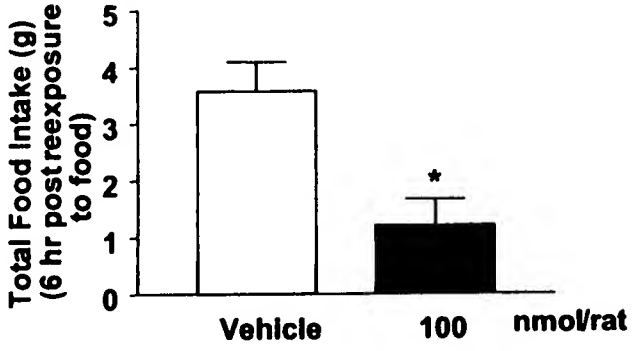
surrounded by photocell, which allowed for automated recording of motor activity. Animals were under no motivational constraints and were free to move around the cage.

Male Sprague-Dawley rats (n = 6-8 per dose) were administered ARE112 intraperitoneally (IP) prior to placement into in locomotor activity cages. Based upon the data, it can be concluded that ARE112 does not affect locomotor activity where animals were exposed to locomotor activity cages for 1.5 hour immediately after injection of ARE112. While the data support the conclusion that ARE112 decreases locomotor activity in rats 16 hours post-injection, thus indicating that ARE112 has some sedative activity, (*e.g.*, the animals appear relaxed and demonstrate little to no anxiety-like behavior), this sedative activity is mild and cannot, in and of itself, account for the decrease in food intake.

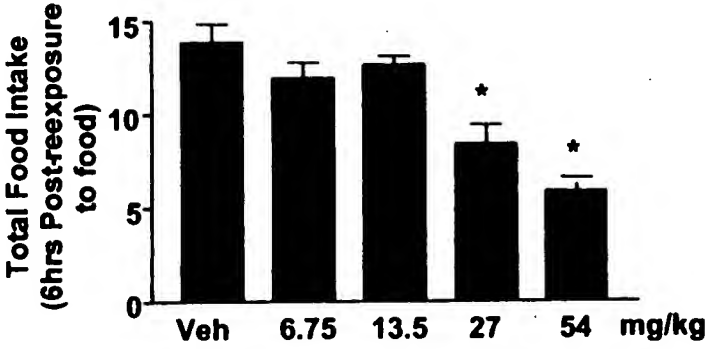
As further evidence of this mild sedative activity, animals were measured to determine their startle reflexes. In this assay, ARE112 was orally administered in non-food deprived rats 4 hours prior to testing. Animals were subjected to a pre-pulse of 12db followed by a 120db pulse and subsequently measured for the height in which the animals jumped. Treated rats at 27 and 54mg/kg did not jump as high, as compared to the vehicle and rats treated at the low dose of 13.5 and 20.25mg/kg, in response to the pulse. This data further suggests that at a higher dosage of 18F112, animals demonstrate a mild sedative activity.

Appendix C12

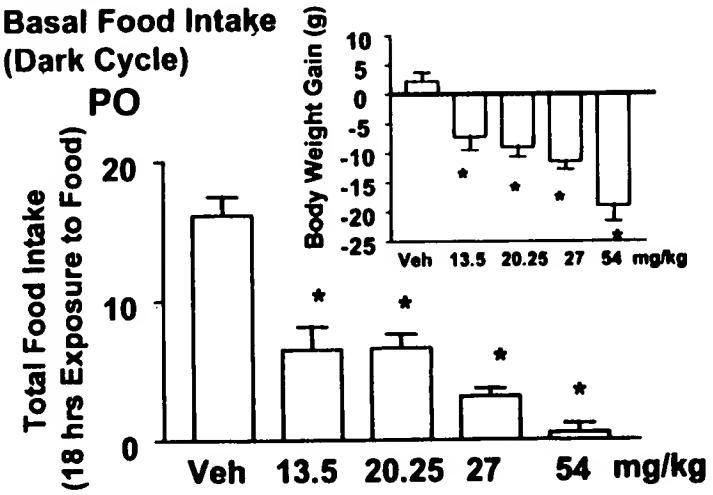
Panel 1
Fasted Rats (24 hr)
ICV



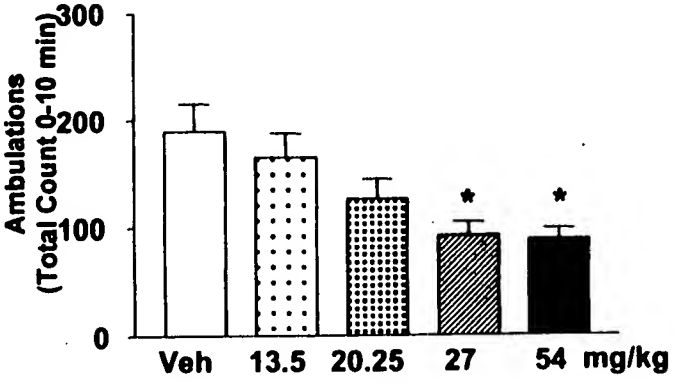
Panel 2
Fasted Rats (24 hr)
PO



Panel 3
Basal Food Intake
(Dark Cycle)
PO



Panel 4
Locomotor Activity



For the food intake studies, ARE112 was administered 2 to 4 hrs p.o or 30 min icv prior testing. For the locomotor activity studies, ARE112 was administered 4 hrs prior testing (no habituation to testing cage, testing during light cycle). *P < 0.05 vs. Vehicle group, Dunnett's test (n=6-8/group).